Myosin–Product Complex and Its Effect on the Steady-State Rate of Nucleoside Triphosphate Hydrolysis*

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ABSTRACT: The kinetic properties of the myosin–product complex were studied by rapid separation on a Sephadex column. In the hydrolysis of [^{3}H,γ-^{32}P-labeled MgATP at pH 8, 20°, both ADP and P_i were found to be bound to myosin and to dissociate at similar rates. When temperature or ionic strength were reduced, ADP dissociated more slowly than P_i. ADP added to myosin dissociated at approximately the same rate as ADP bound as a result of hydrolysis. Dissociation rates were comparable with the steady-state rates of substrate hydrolysis. Similar results were obtained with MgITP as substrate.

When the column buffer contained unlabeled ATP or ADP, the rate of ADP dissociation was increased. The results are interpreted by a mechanism in which the rate-limiting step in hydrolysis is product dissociation and the two sites on the enzyme interact.

The early phase of hydrolysis of ATP by myosin is characterized by a rapid "early burst" of phosphate production in which the actual rate of hydrolysis for the first molecule of ATP is the order of 50–75 sec^{-1}, a value which exceeds the steady-state rate by a factor of 50–1000 (Lymn and Taylor, 1970). As a consequence, the enzyme is expected to be present in the steady state mainly as an enzyme–product complex and evidence has been presented by Tonomura and collaborators that a phosphorylmyosin accounts for at least part of the product complex. However, attempts to isolate a phosphorylmyosin have not been successful and Sartorelli *et al.* (1966), basing their conclusions on ^{18}O-exchange measurements, have argued against the most likely types of phosphoryl complexes. Tonomura has also concluded that ADP is not bound in the steady state (Imamura *et al.*, 1966) and that the myosin–phosphate complex decays too slowly to account for the steady-state rate. Consequently the pathway of hydrolysis was assumed to proceed largely by a second route in which the myosin–phosphate complex was not formed.

We have investigated the rate of decay of the myosin–product complex under a variety of conditions of ionic strength, temperature, and pH by means of rapid gel filtration on columns of Sephadex G-25. Both ADP and phosphate were bound to the enzyme and the rate of decay was found to correspond to the steady-state rate. Although the interpretation is somewhat complicated by occurence of what we have termed site interaction, the results can be accounted for by a simple model in which the dissociation of products is the rate-limiting step, and in which the sites are equivalent. No evidence was obtained in support of an acid-labile phosphorylmyosin and we regard the product complex as a simple association, comparable with the binding of ADP alone.

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Materials and Methods

The preparation of myosin, heavy meromyosin, and many of the methods employed have been described previously (Lymn and Taylor, 1970). ^{3}H-Labeled ATP and ADP and ^{14}C-labeled ITP were purchased from Schwarz.

Column Chromatography. All experiments were performed using 2.5 × 30 cm Glenco columns, eluted by means of Holter or Harvard Apparatus pumps connected to the inflow end. Columns were thermostated by circulating water from a Forma bath through a glass jacket (Glenco). The solvent reservoir was also placed in the temperature bath. In the standard experiment a 6–7-cm bed of Sephadex G-25 fine was employed and the remaining 24 cm was filled with elution buffer. A 0.8-ml sample containing radioactive nucleotide in 2.5% sucrose was layered on top of the resin, in the basket provided by the manufacturer, using a plastic syringe and a length of Tygon tubing enclosed in glass for rigidity. The lower stopcock was opened and 2.5–3 ml of buffer were allowed to flow under gravity to bring the nucleotide sample just inside the resin bed. (Layering was checked occasionally by including *o*-cresolsulfonephthalein indicator in the sample.) A 0.8-ml sample of myosin was then layered on top of the resin in 2.5% sucrose. The column was immediately closed and elution was begun. The 24-cm layer of solvent prevented disturbance of the sample zone by the high flow rate of the input buffer. Aliquots of 5 ml were collected by hand in calibrated tubes and the time from starting elution to collection of the last tube was measured on a stopwatch. The flow rate was constant and could be adjusted reproducibly by varying the speed setting of the pump. Flow rates up to 0.6 ml/sec were employed.

In some experiments the device shown in Figure 1 was used; a simple T mixing chamber was constructed from a Lucite block with Teflon valves. The samples in 2.5% sucrose were layered at the bottom of each cylinder. A small air hole in each cylinder allowed the pistons to be inserted to the buffer level before starting the run. Enzyme and substrate were placed in positions 1 and 2, respectively, and mixed by manually depressing the block. The solution traveled around the

loop of polypropylene tubing, mixed with the contents of position 3, and entered the column. The three-way Teflon stopcock was turned from the sample inlet to pump inlet position (the pump was turned on just before mixing the samples). The column was packed under pressure to just below the top; the standard fitting was modified to take a baffle plate about $^1/_8$ in. thick, which served to disperse the influent stream. Peaks were less well resolved than with the layering technique but the mixing device allowed experiments in which another component was added to the myosin–product complex.

Analysis of Column Effluent. In most experiments myosin was allowed to react with [^{3}H,γ-^{32}P]ATP, and the distribution of myosin, ATP, ADP, and phosphate was determined. From each 5-ml fraction a 1-ml aliquot was taken and the protein peak was located by measuring OD_{276} (the nucleotide concentration was too low to interfere with this measurement). To the aliquot, 10 ml of Bray's solution (Bray, 1960) was added and total radioactivity, ^{3}H and ^{32}P was determined using a Packard scintillation spectrometer. The contribution of ^{32}P to counts in the ^{3}H channel was less than 5% while ^{3}H made a negligible contribution to the counts in the ^{32}P channel. The remainder of the sample was used for the determination of phosphate, ADP, and ATP. Phosphate was measured by the technique described previously (Lymn and Taylor, 1970). The charcoal filter pad containing the adsorbed nucleotides was shaken in 4 ml of 50% ethanol–1% ammonia; charcoal was removed by filtration on a 2.5-cm Millipore filter (0.45 μ pore size), and an aliquot of the filtrate was counted to determine ^{3}H and ^{32}P content of the nucleotide fraction.

Since the radioactivity measurements were made in different solvents and with different counters, the counting efficiency was determined for each type of measurement. A 20-μl aliquot of the sample to be applied to the column was spotted on a Millipore filter and counted in the end-window counter to determine total available ^{32}P. A second 20-μl aliquot was diluted to 1 ml with column buffer and counted in the scintillation counter to calibrate the total radioactivity measurement. A third sample was carried through the separation procedure to calibrate the charcoal–nucleotide determination.

The measurement of total radioactivity per tube, after scaling, gives the sum of [^{32}P]P_i, [^{3}H,^{32}P]ATP, and [^{3}H]ADP. The phosphate precipitation gives [^{32}P]P_i directly. The charcoal–nucleotide radioactivity yields ATP concentration from from the ^{32}P counts and ADP from the difference of ^{3}H and ^{32}P counts, *i.e.*, ^{3}H radioactivity is the sum of [^{3}H]ADP and [^{3}H,^{32}P]ATP. Total ^{32}P count − [^{32}P]P_i also gives ATP and (total ^{3}H − (total ^{32}P − [^{32}P]P_i)) gives ADP. Thus the concentrations of ATP and ADP can be calculated in two different ways which serves as a check on the calibration procedure. By this technique the distribution of protein, ATP, ADP, and P_i could be rapidly determined on the large numbers of samples generated in the column experiments.

In tubes containing both myosin and ATP, hydrolysis will continue after the sample is collected. Therefore experiments were included in which the effluent was collected in tubes containing 1.5 ml of cold 2 N perchloric acid plus unlabeled ATP, ADP, and P_i as carrier. In these experiments the protein profile could not be estimated but this was not a serious objection as peak positions for successive runs on the same column were reproducible.

Equilibrium Dialysis. Samples of 0.5- or 1.0-ml volume were dialyzed against 10 ml of the appropriate solution, in stoppered test tubes, thermostated in a circulating-water bath (Forma). Dialysis reached equilibrium in 8–10 hr at 20° or overnight (*ca.* 18 hr) at 0°. Aliquots of 0.2 ml of the sample and dialysate were added to counting vials, containing 1 ml of Hyamine 10× (Packard Instruments), incubated at 60° for 1 hr, and 10 ml of Bray's solution was added. The protein was dissolved by Hyamine treatment and the resulting sample after addition of scintillation solution was homogeneous.

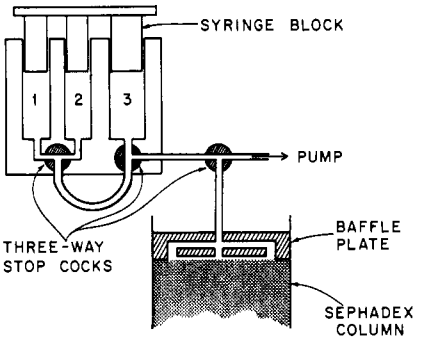


FIGURE 1: Mixing device. Samples are layered under buffers in positions 1, 2, and 3. Mixing takes place in two three-way stopcocks as syringe block is forced down. Column is filled with Sephadex to level of baffle plate. The apparatus is constructed from Lucite with Teflon stopcocks and Tygon tubing connections.

Results

Half-Life of Enzyme–Product Complex. The half-life of the myosin–products complex was determined by chromatography on a G-25 (fine) Sephadex column. Approximately 0.8 ml of [^{3}H,^{32}P]ATP in 2.5% sucrose was layered on top of the resin bed and 3–3.5 ml of buffer was allowed to flow through the column to bring the ATP zone into the resin bed. Approximately 0.8 ml of myosin in 2.5% sucrose was then layered on top of the resin bed and the column was immediately eluted and 5-ml fractions were collected by hand.

Previous results have established that the first two steps in the mechanism are

$$M + XP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} MXP \xrightarrow{k_2} M\cdot X\cdot P$$

where XP refers to a nucleoside triphosphate; the nature of the M·X·P complex is not specified. $k_1 \sim 5 \times 10^5$ M^{-1} sec^{-1} and $k_2 \sim$ 50–75 sec^{-1} (Lymn and Taylor, 1970). In most experiments the concentrations of ATP and myosin were 0.5–2×10^{-5} and 0.6×10^{-5} M, respectively. Steps one and two will be completed in about 1 sec, thus as the myosin zone crosses the ATP zone on the column it is converted into the M·X·P state. In various experiments, columns were run at flow rates ranging from 7.5 to 35 sec per 5-ml fraction, consequently the zones are in contact for at least 5 sec. The M·X·P complex will separate from the remaining XP, and decay to myosin plus products as it migrates down the column. Since the time necessary to elute the myosin is comparable to the expected half-life of the complex, one should obtain a peak of radioactivity whose leading edge is coincident with the myosin peak, a tail of radioactivity, and a second peak due to free nucleosides and phosphate. A typical column profile is

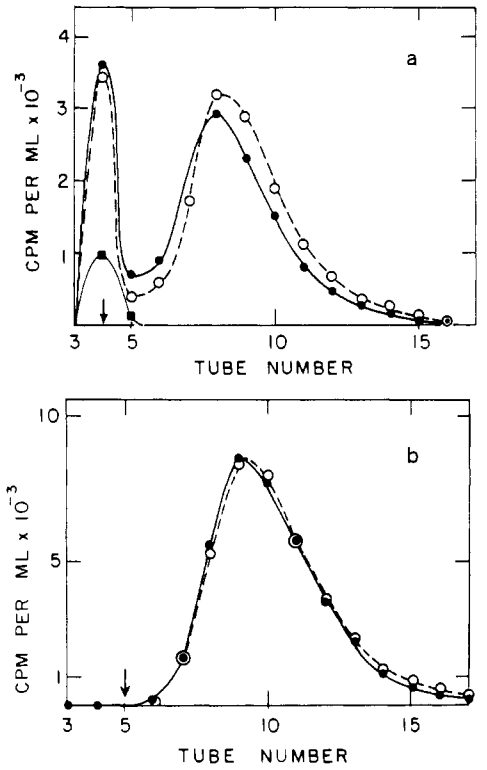


FIGURE 2: Elution profiles. (a) Of myosin from Sephadex G-25 fine column 6 × 2.5 cm at room temperature. Input 0.8 ml of 10^{-5} M [^{3}H,^{32}P]ATP, 0.8 ml of myosin 0.6 × 10^{-5} M. Both samples were layered on top of the column bed in 2.5% sucrose. Samples and eluting buffer contained 0.5 M KCl–Tris buffer (pH 8)–10^{-3} M $MgCl_2$. Samples (5 ml) were collected at a flow rate of 12 sec/tube. (■—■) Myosin concentration, OD_{276}; (●—●) ^{3}H concentration; (○—○) ^{32}P concentration. Ratio of ^{3}H and ^{32}P counts per minute was determined on an aliquot of the input sample and actual counts of the sample tubes were scaled. (b) From the same column as in part a with the myosin sample omitted. Arrow indicates the approximate position at which myosin would elute from this column. Flow rate 11 sec/tube. In column a the ATP sample was brought into the bed by allowing 3 ml of buffer to pass over the column under gravity. In column b this step was omitted; consequently the peak position is displaced by approximately half a tube compared with column a.

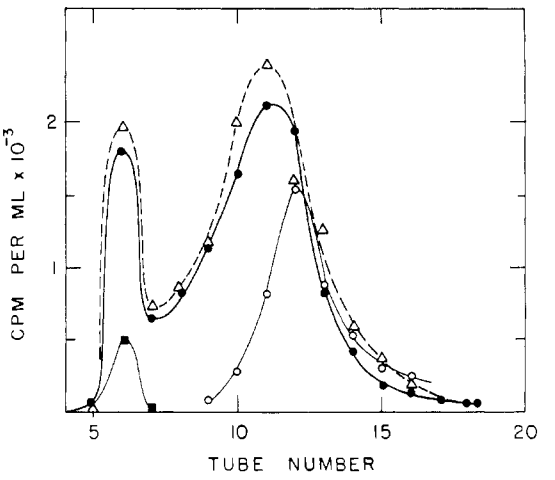


FIGURE 3: Elution profile of myosin, ATP, ADP, and phosphate from Sephadex G-25 fine. Conditions as in Figure 2. Counts per minute have been scaled by calibration of counting efficiency such that radioactivity is proportional to concentration for each species. (■) Myosin, (●) phosphate, (△) ADP, and (○) ATP. Flow rate, 17 sec/tube.

shown in Figure 2a which indicates that an appreciable fraction of the radioactivity is still bound after the 50 sec required to elute myosin in this experiment. Figure 2b shows the distribution of radioactivity when the column is eluted in the absence of myosin.

The distribution of P_i, ADP, and ATP, was determined by the method described in Materials and Methods. Results of a typical experiment are shown in Figure 3. Under the conditions employed (pH 8, 0.5 M KCl–10^{-3} M $MgCl_2$, 24°), nearly equal amounts of ADP and P_i remain bound to myosin. The myosin peak is clearly resolved from free ATP and there is a tail of radioactivity for both products. The total amount of ADP and P_i produced was the order of 1 mole/mole of myosin. In other experiments the effluent was collected in tubes containing 1.5 ml of 2 N perchloric acid plus ATP, ADP, and P_i as carrier in order to stop any reaction in the tubes containing myosin. The results were essentially the same.

Therefore both reaction products remain bound to the enzyme under these conditions and dissociate at approximately the same rate. The rate constant of dissociation or half-life of the product–complex can be estimated from the ratio of bound P_i and ADP to total P_i and ADP, and the time from separation of the zones to elution of the myosin peak. However, a second method was used when possible. We note that the radioactivity in tubes 5–11 was initially bound to myosin and as elution proceeds, product dissociates and is left behind. Thus, ignoring spreading due to diffusion, a given amount of radioactivity dissociates in a 12-sec interval and is eluted in tube 11, a smaller amount dissociates in the next 12 sec and is eluted in tube 10, etc., until finally tube 6 is eluted which contains undissociated radioactivity. We therefore sum the radioactivity of tubes 6–11 and make a semilog plot *vs.* tube number (or time, since the flow rate is constant). Figure 4 shows a plot of the data from Figure 3. The plot is linear and the half-life of the complex is obtained from the slope. This procedure is superior to calculating the half-life from the bound fraction because it makes better use of the data and, since the process is an exponential decay, any portion of the decay curve can be employed. It is not necessary to know the time lag for separation of the zones; the result would not be affected by any overlap in layering of the zones. If myosin elutes in more than one tube, as was often the case, the counts from these tubes are combined for the final point of the plot.

To estimate the limitation on the method because of diffusion spreading, a semilog plot of the profile for elution of [^{3}H,^{32}P]ATP in the absence of protein (as in Figure 2b) was made. Since this profile is Gaussian, *i.e.*, the form is e^{-ax^2}, the logarithmic plot shows downward curvature and the apparent half-life is the order of 5–10 sec. Thus the calculation procedure is applicable only to half-lives greater than about 15 sec. Experiments were rejected if log plots showed downward curvature.

In some experiments at low ionic strength there was a tailing or holdup of the free ADP peak, possibly due to weak interaction with Sephadex. In such cases the half-life was estimated from the bound fraction. (This effect can be seen in Figure 11.)

As a further control on the use of the column procedure, myosin was chromatographed in the presence of 10^{-2} M

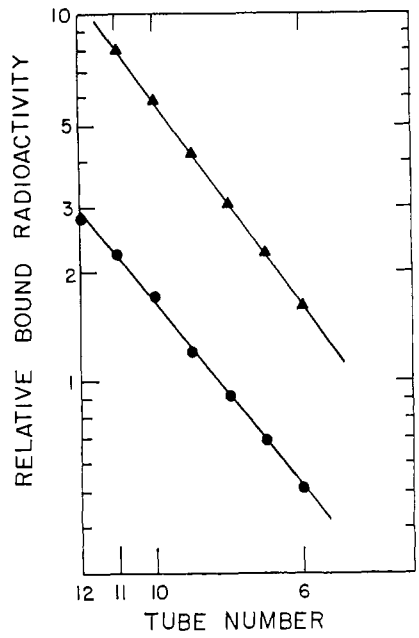


FIGURE 4: Semilog plot of bound radioactivity *vs.* tube number. (▲–▲) Phosphate and (●–●) ADP. The points were obtained from data shown in Figure 2 by the method described in the text. Flow rate 17 sec/tube. The two curves have been displaced for purposes of illustration.

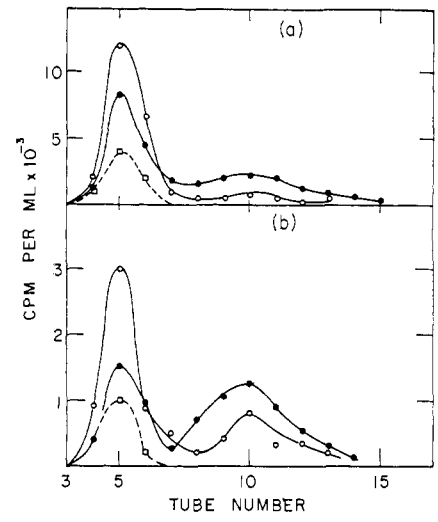


FIGURE 5: Elution profile of myosin, ADP and phosphate at 10 and 0°. Input 0.8 ml of [^{3}H,^{32}P]ATP, 10^{-5} M; 0.8 ml of myosin, 0.6×10^{-5} M. Samples and column buffer contained 0.5 M KCl–2×10^{-3} M $MgCl_2$–Tris buffer (pH 8). (□) Myosin, (●) phosphate, and (○) ADP. (a) 10°, 12 sec/tube, (b) 0°, 30 sec/tube.

$CaCl_2$ or 10^{-3} M EDTA. Since the steady-state rate is much larger we expect little or no radioactivity bound to myosin. In the experiment employing $CaCl_2$ as activator, approximately 1 mole of P_i was liberated per mole of myosin but less than 0.2% of the radioactivity was present in the myosin tubes and this residual was due mainly to a diffusion tail. Log plots in both cases showed downward curvature and closely approximated the profile for free nucleotide. When myosin was incubated with ATP in the presence of 10^{-3} M $MgCl_2$ for 1 hr before running the column, very little radioactivity was obtained in the myosin peak and was attributable to binding of a small amount of ADP.

The results for MgATP as substrate fitted a single exponential decay of the product complex and if this is the rate-limiting step in the hydrolysis reaction then $0.69/t_{1/2}$ should equal k, the steady-state rate constant for hydrolysis. The results of twelve determinations at 24°, pH 8, 10^{-3} M $MgCl_2$–0.5 M KCl, where $t_{1/2} = 31.5 \pm 3$ sec for ADP and $t_{1/2} = 30 \pm 3$ sec for phosphate. The error quoted is the standard error of the mean. Thus the value of k is 0.023 ± 0.003 sec^{-1} determined from the decay data, while the steady-state rate was 0.025 ± 0.005 sec^{-1} for various myosin preparations. The rate constant determined by the columns procedure is a first-order decay constant and does not depend on the molecular weight of the site. The steady-state rate assumes a molecular weight of 5×10^5 daltons and since there may be two hydrolytic sites per molecule, the rate per site could be one-half as large as the figure quoted if the sites are identical and independent. Thus the results support the conclusion that the rate-limiting step is the decay of the enzyme–product complex but in view of experimental errors (about ±10%) as well as uncertainty in molecular weight of the site, we cannot rule out a discrepancy of a factor of 2. The latter problem will be considered in more detail below.

Effects of Temperature, pH, and Ionic Strength on Half-Lives. The rates of dissociation of both products were equal within experimental error at pH 8, 0.5 M KCl, and room temperature. As this finding could be fortuitous, an investigation was made of the effects of varying temperature, pH, and ionic strength on the decay rates of the two products. At lower temperatures (at pH 8 and 0.5 M KCl) the decay of ADP was clearly slower than that of phosphate. Figure 5a,b shows results at 10 and 0°. At 10° the two rates are significantly different, while at 0° the difference is even more pronounced. In the example shown in Figure 5a, the column was run at a flow rate of 12 sec/tube, which is satisfactory for measurement of the phosphate half-life, but the fractional decay of ADP is small and any error in calibration of counting efficiency leads to large error in half-life, since ADP is determined by difference. ADP decay can be measured at a flow rate of 30–40 sec/tube (Figure 5b). Because of the very slow decay, an alternate method was also used for ADP measurements at 0°.

Myosin and ATP were mixed at 0°, layered on the column and 10 ml were eluted to separate myosin–product from ATP zones. Flow was stoppered for 0, 5, or 10 min to allow dissociation to proceed, then the myosin was eluted. Decay time was determined from the slope of a semilog plot of radioactivity in the myosin peak *vs.* time. This procedure yielded values for the decay time of the same magnitude as those obtained by the standard column procedure.

A plot of the half-lives and of steady-state rate of hydrolysis expressed as $0.69/k$ is shown in Figure 6. Although the experimental errors are large, it is clear, at least qualitatively, that as temperature is lowered, decay of ADP becomes slower than phosphate. It may also be slower than the steady-state rate, but it cannot be concluded that the difference is significant because of the uncertainty in the number of active sites.

The effect of pH was investigated in the range from 5.5 to 9.0 in Tris-acetate buffers, 0.5 M KCl, and 20°. The steady-state rate yields a minimum at pH 7.5 but since the ratio at 7.5 to 5.5 or 9 is only about a factor of 2, the variation in

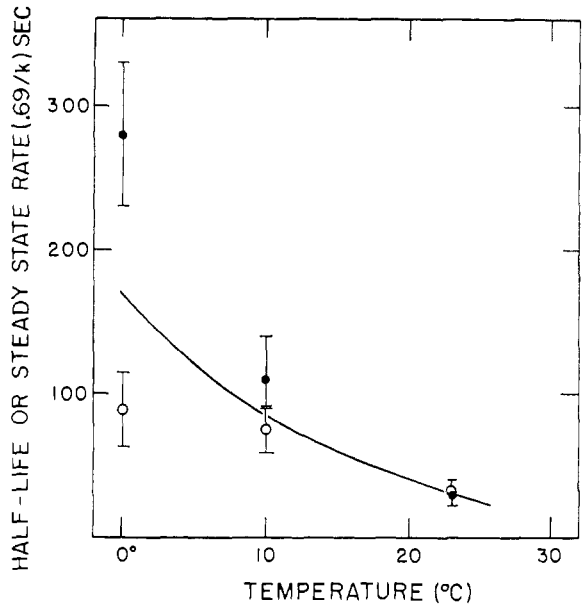


FIGURE 6: Comparison of half-life of enzyme–products complex with steady-state rate expressed at $0.69/k$ as a function of temperature. Conditions are 0.5 M KCl–2 × 10^{-3} M $MgCl_2$–Tris buffer (pH 8). (⊢○⊣), phosphate half-life; (⊢●⊣), ADP half-life; solid line, $0.69/k$, where k is the steady-state rate constant in sec^{-1}. The error bars indicate the standard deviations of means of three or more determinations.

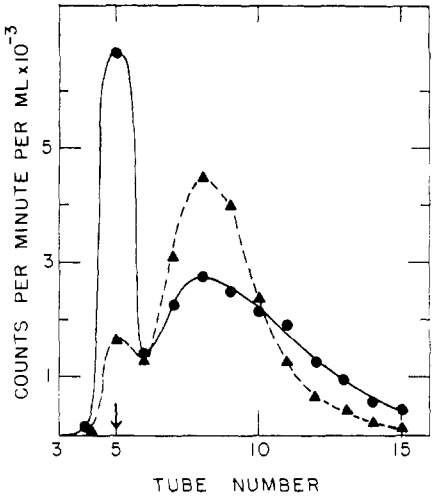


FIGURE 8: Elution profile for heavy meromyosin, ADP, and phosphate at 10°. Input: 0.8 ml, [^{3}H,^{32}P]ATP, 10^{-5} M; 0.8 ml of heavy meromyosin, 0.5 × 10^{-5} M. Samples and column buffer contained 0.05 M KCl–2 × 10^{-3} M $MgCl_2$–Tris buffer (pH 8). (●–●) ADP and (▲---▲) phosphate. Myosin peak is indicated by the arrow. Flow rate is 15 sec/tube.

decay constants with pH would be difficult to detect. However, a moderate change in relative rates for the two products could probably be detected and in view of speculation on possible phosphorylated intermediates which might show different dependence of stability on pH, it was necessary to investigate the decay rates.

The points in Figure 7 are the decay rates ($0.69/t_{1/2}$) while the solid curve is the steady-state rate. There was no evident trend in rate with pH. The means of all determinations were 32 ± 2 and 28 ± 2 sec for ADP and phosphate, respectively. Since these standard errors are no larger than for the set of determinations at pH 8, the data do not allow a distinction to be made between a shallow minimum *vs.* a constant pH dependence of the rate of decay.

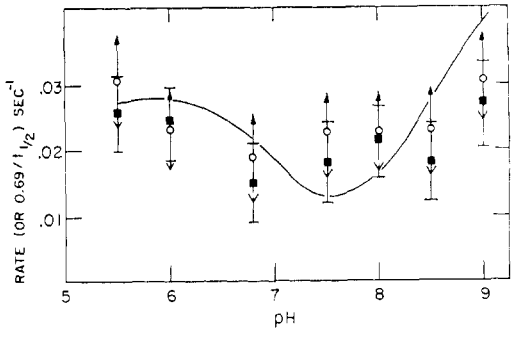


FIGURE 7: Comparison of the steady-state rate of hydrolysis as a function of pH, with half-life of enzyme–product complex. 0.5 M KCl–2 × 10^{-3} M $MgCl_2$–(20°)–0.05 M Tris-acetate buffer. (←○→) Phosphate half-life, (⊢■⊣) ADP half-life. Solid curve is the steady-state rate (sec^{-1}). The half-lives are plotted as $0.69/t_{1/2}$. The error bars indicate standard errors of means of two or more experiments.

The dependence of rate of decay on ionic strength was investigated using heavy meromyosin at pH 8 and various temperatures (24, 20, 10, 0°). The steady-state rate increases with decreasing ionic strength and becomes too fast to be measured satisfactorily by the column procedure at room temperature. Qualitatively, as shown in Figure 8, the ADP decay appears to be slower than phosphate. A plot of the ionic strength dependence at 10° is shown in Figure 9. The P_i dissociation appears to parallel the change in steady-state rate but ADP dissociation is clearly slower than the steady-state rate, particularly at the lowest ionic strength.

Binding of [^{14}C,^{32}P]ITP was investigated in a small number of experiments at pH 6.0. The higher rate and lower binding constant made quantitative measurements difficult but at 0° a half-life of 50 sec was obtained for P_i. Thus product dissociation is probably the rate-limiting step for this substrate, but the kinetics were not investigated in detail.

Finally, dissociation of myosin–ADP was investigated by incubation of myosin with ADP, and elution from the column. Since experiments described above indicated that under some conditions the dissociation of ADP was the rate-limiting step in hydrolysis, it could be asked whether rate of dissociation of ADP formed on the enzyme surface by hydrolysis was very different from dissociation of a bound ADP. The experiment is subject to larger errors because the half-life was estimated from the fraction of ADP remaining bound when the peak was eluted, while the initial binding depends on the association constant and the number of sites. In some experiments the myosin was dialyzed overnight against ADP, and the amount of bound radioactivity determined on an aliquot of the sample. Figure 10 shows that dissociation of ADP is slow enough to be measured under the same conditions as the hydrolysis experiments.

The ratio of initial concentration of heavy meromyosin to ADP was approximately 1 to 2 on a molar basis; in 0.05 M

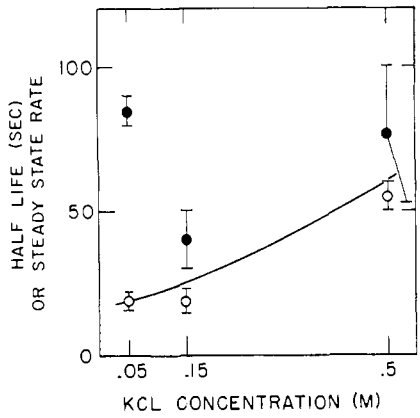


FIGURE 9: Comparison of half-life on enzyme–products complex with steady-state rate for heavy meromyosin as a function of ionic strength (10°). (⊢○⊣) Phosphate half-life; (⊢●⊣) ADP half-life; solid curve is steady-state rate expressed as $0.69/k$. Error bars are standard errors of means of two or more experiments. Conditions are 2×10^{-3} M $MgCl_2$–Tris buffer (pH 8).

KCl at 10°, the bound peak in Figure 10 corresponds to 0.06 mole/mole. Estimating the initial bound ADP from the data of Lowey and Luck (1969) ($K = 1\text{–}2 \times 10^5$ M^{-1}, one to two sites per molecule), the calculated half-life would range from 16 to 21 sec. Based on the fraction bound at zero time, as determined by dialysis, the half-life was 15–25 sec. This result is about one-third as large as the half-life obtained in ATP hydrolysis experiments but it is certainly the right order of magnitude.

Effect of High Nucleotide Concentration on the Dissociation of the Product Complex. The rate of dissociation of products appeared to approximately parallel the steady-state rate for the conditions of pH, temperature, and ionic strength investigated. However, at low temperature and low ionic strength, ADP dissociation was slower than P_i dissociation, but in addition it was slower than the steady-state rate, even allowing a factor of two for the number of sites. The column experiments were performed at a low ATP concentration, and an ATP to myosin mole ratio of less than 2 to 1. Consequently the decay rate measured on the column was mainly from a state in which only 1 mole of product was bound, while the steady-state rate was determined by extrapolation to high concentration, and the discrepancy might be due to interaction between sites.

To investigate this effect, the mixing device shown in Figure 1 was used. Myosin (10^{-5} M) was mixed with [^{3}H,^{32}P]ATP (10^{-5} M). After passage around the loop of tubing, it was further mixed with 10^{-3} M unlabeled ATP and eluted through the column with a buffer containing 10^{-3} M ATP. All solutions also contained 2×10^{-3} M $MgCl_2$. Thus a myosin–products complex was first formed with one site containing labeled products. The complex was then allowed to decay with the second site occupied by unlabeled substrate or product. As shown in Figure 11, the ratio of ADP to P_i bound to myosin is two in the control and approximately one when ATP was present. In both experiments, the phosphate half-life of 40 sec was in reasonable agreement with the steady-state rate. Loading with 10^{-3} M ADP in the column buffer also increased the rate of ADP dissociation from the enzyme, though it did not raise the rate as much as ATP.

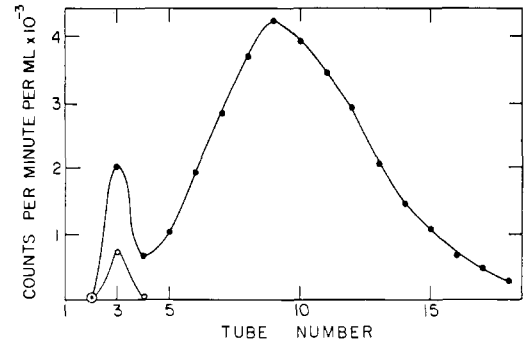


FIGURE 10: Elution profile of heavy meromyosin and ADP (10°, 0.5 M KCl–2×10^{-3} M $MgCl_2$–Tris buffer, pH 8). Heavy meromyosin (10^{-5} M) and [^{3}H]ADP (2×10^{-5} M) were incubated at 10°, layered on the column and eluted at a flow rate of 15 sec/tube. (○–○) Myosin and (●–●) ADP.

Discussion

Under a variety of conditions of pH, temperature, and ionic strength, the hydrolysis of ATP by myosin in the presence of Mg ion leads to a relatively stable myosin–ADP–P_i complex. The rate of decomposition of this complex is approximately equal to the steady-state rate of hydrolysis under the conditions investigated. Therefore it is concluded that dissociation of the product complex is the rate-limiting step, and to a first approximation, the mechanism can be represented by

$$M + ATP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} M \cdot ATP \xrightarrow{k_2} M \cdot ADP \cdot P \xrightarrow{k_3} M + ADP + P_i \quad (1)$$

It was shown previously (Lymn and Taylor, 1970) that k_2 is the order of 50–75 sec^{-1} while k_3 is 0.02 sec^{-1} at 20°, pH 8, and 0.5 M KCl. Although it will be necessary to consider effects

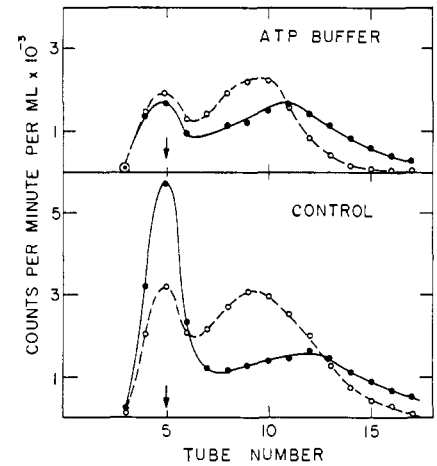


FIGURE 11: Effect of ATP on rate of dissociation of heavy meromyosin–ADP–phosphate complex. Conditions 10°, 0.05 M KCl–Tris buffer (pH 8)–2×10^{-3} M $MgCl_2$. Heavy meromyosin (10^{-5} M) was mixed with [^{3}H,^{32}P]ATP (10^{-5} M) in the device illustrated in Figure 1. After a few seconds it was further mixed with column buffer (control), or with 10^{-3} M ATP, and applied directly to the column and eluted with plain buffer (control) or buffer plus 10^{-3} M ATP. (●–●) ADP and (○–○) phosphate. Arrow indicates position of myosin peak.

which complicate this simple mechanism, some general properties will be discussed first. The column procedure allows the decay constant to be measured without stopping the reaction with acid. Consequently there is no reason to invoke a phosphorylated intermediate, which is acid labile (Kanazawa and Tonomura, 1965), in order to explain the early burst phenomenon. Within the experimental error of ±30% the decay rate did not depend appreciably on pH in the range from 5 to 9. The early burst is simply a consequence of the large value of k_2 compared with k_3.

While previous workers have concentrated on a myosin–phosphate intermediate (Nakamura and Tonomura, 1968), our results indicate that ADP is also bound and the interaction of myosin with ADP is probably the property which controls the steady-state rate. In this connection it is of interest to compare the steady-state rates with the myosin–ADP binding constants, in the presence of either Mg or Ca as the divalent ion. The rate constants k_1 and k_2 are very similar in the presence of either Ca or Mg (Lymn and Taylor, 1970), consequently the difference must be in k_3. With Ca ion present the rate of dissociation of the product complex is too fast to be measured by the column procedure, which is consistent with the interpretation that the 20–50-fold increase in steady-state rate is due to a faster rate of dissociation of Ca–myosin–ADP complex. Since the equilibrium constant for binding of MgADP is about 20 times larger than for CaADP (Lowey and Luck, 1969), the difference in the rates of hydrolysis roughly parallels the difference in the binding constants.

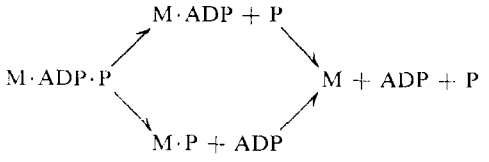
Morita's (1969) recent study on the spectral shift induced in heavy meromyosin by either ATP or ADP, shows that the induced shift decays at about the same rate as the steady-state rate. Since phosphate alone does not cause this shift, her results support a myosin–ADP complex, and provide an alternate method for measuring the rate of dissociation.

A role for the nucleotide in determining the rate has been suggested by Blum and Felauer (1959), and Kiely and Martonosi (1968). Eigen and Hammes (1963) pointed out that the different rates for the dissociation of Mg and Ca from nucleotides might influence enzyme rates, though the rate constants to which they referred are both too large to apply here. Nonetheless, a similar concept is applicable to dissociation of the enzyme–metal–nucleotide complex.

If product dissociation is the rate-limiting step, differences in rates of hydrolysis of nucleoside triphosphates must be attributed to differences in stability of the myosin–nucleoside diphosphate complexes. ITP was previously shown to undergo a rapid hydrolysis step (Lymn and Taylor, 1970), though the apparent rate constant for binding was less than with ATP, and a slow dissociation of the product complex was found. Much has been made of differences in shape of pH–rate curves for ITP *vs.* ATP and of the effect of titration of SH groups on the pH curve for ATP (Rainford *et al.*, 1964). Although we cannot rule out the possibility that these differences are due to changes in enzyme configuration, the experimental results could be simply explained by differences in binding of the nucleoside diphosphates.

The rate of dissociation of the complex formed by incubation of myosin with ADP is comparable with dissociation of the enzyme–product complex produced by ATP hydrolysis. Since, in the first instance the ADP–myosin linkage is not likely to be a covalent bond, and the dissociation of the ADP–myosin complex accounts for the early burst phenomenon, there appears to be no need to postulate a phosphorylated intermediate to explain any of the properties of the enzyme.

In general, dissociation of the products complex would require specification of four rate constants



Several lines of evidence suggest that the rate is controlled by the ADP dissociation step: both products dissociate at comparable rates for various conditions of pH, ionic strength, and temperature: when a distinction could be made it was the ADP which dissociated more slowly; the myosin–ADP complex dissociates at a rate similar to the complex formed by hydrolysis, phosphate is probably only weakly bound to myosin (a value for the phosphate binding constant does not seem to be available but weak binding may be inferred from the lack of competitive inhibition by phosphate). It is likely that M·P undergoes rapid dissociation once the nucleotide comes off.

An ADP or ATP complex with a very long half-life has been reported by Burton and Lowenstein (1964) and Szent-Györgyi (1968). However, the value for the half-lives reported by these authors considerably exceeds the maximum half-life we observed, of about 5 min.

At low temperature and low ionic strength the rate of ADP dissociation is slower than P_i dissociation but it is also slower than the steady-state rate. Because of experimental errors as well as uncertainty in the number of hydrolytic sites, the discrepancy might not be significant. However, relative rates of dissociation are more accurately measured and a direct experiment showed that the rate of ADP dissociation increased and became essentially equal to the P_i rate when decay was allowed to proceed in presence of 10^{-3} M ATP or ADP, *i.e.*, when the second site was occupied by substrate or product. One must consider the possibility that this apparent increase in rate is caused by suppression of a back-reaction, *i.e.*, the recombination of myosin with free ADP. Consideration of free ADP concentration in the column (less than 10^{-7} M), the magnitude of the ADP binding constant (10^5 M^{-1}), and of the rate constants for association and dissociation, makes this possibility very unlikely.

Previously, in discussing kinetic studies of the transient state, it was suggested that an interaction between sites which reduced the rate of binding and hydrolysis at one site if the other was occupied was the simplest mechanism that could account for the data. In line with this suggestion the rate of dissociation of product at one site might be increased if the other site is occupied by substrate or product.

If interaction occurs for the binding of ADP as well as ATP, the rate of binding is expected to decrease and of dissociation to increase with increasing ADP concentration, consequently two apparent association constants might be observed.

Recent studies by Lowey and Luck (1969) on heavy meromyosin in 0.05 M KCl yielded an association constant of 10^5 M^{-1} and approximately 1.5 binding sites. The range of free ADP concentrations employed was less than a factor of 5 and the largest concentration was about 5×10^{-5} M. Experiments

over a wider range extending to higher concentrations are necessary in order to detect possible curvature in a Scatchard plot, but experimental errors become very large at high concentrations. Preliminary studies yielded an association constant of 10^5 M^{-1} and 1.3–1.4 sites for concentrations below 10^{-4} M in agreement with Lowey and Luck, but at higher concentrations the data appeared to deviate from a straight line indicating the presence of a second binding site with lower affinity. Because of experimental difficulties we regard these results as suggestive but not conclusive and further studies will be required to settle this question.

The experiments do not provide evidence on the nature of the "interaction" between occupied sites. A direct electrostatic interaction may be adequate to account for the effects described. It is of course an interesting possibility that interaction between occupied sites could bring about a configuration change (which reduces the interaction by moving the sites apart).

The results presented here may appear to be in disagreement with the conclusions drawn by Tonomura and collaborators. Direct measurements of phosphate release (Imamura *et al.*, 1965) gave a value about five times slower than the steady-state rate. These results are based on washing a myosin precipitate which, in our hands, led to serious adsorption problems. ADP dissociation was followed by coupling to NADH oxidation *via* pyruvate kinase and lactic dehydrogenase. Although no "burst" of ADP release was found it was concluded from an indirect argument that ADP dissociation was sufficiently fast so as not to be bound in the steady state (Imamura *et al.*, 1966). In later work a value for the rate of dissociation was given which differed by less than a factor of 2 from the steady-state rate (Kinoshita *et al.*, 1969) and as discussed above conclusions cannot be drawn if the discrepancy is a factor of 2. Rate of ADP dissociation in the steady state was not studied by us except in the case of columns run in the presence of 10^{-3} M ATP (unlabeled). The rate of dissociation was increased but the value corresponded to the steady-state rate of hydrolysis. Tonomura also presented evidence for a slow rate of H^+ liberation in experiments in which 1 mole of ATP was added per mole of myosin. The rate was very much less than the steady-state rate and the result was interpreted as proton liberation which paralleled phosphate release (Imamura *et al.*, 1965). However, in a later study (Tonomura *et al.*, 1969) under similar conditions, this slow H^+ step was at least five times faster and did not differ by more than twofold from the steady-state rate.

Considering the more recent work, the experimental evidence obtained by Tonomura is not very different from the results presented here. The interpretation is certainly different and more complex. Although it is doubtful that a single-site mechanism is capable of explaining all of the results it is not appropriate to argue the relative merits of more complex schemes at this stage.

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References

Blum, J. J., and Felauer, E. (1959), *Arch. Biophys. Biochem. 81*, 285.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Burton, P., and Lowenstein, J. M. (1964), *Biochem. J. 90*, 7c.

Eigen, M., and Hammes, G. G. (1963), *Advan. Enzymol. 25*, 1.

Imamura, K., Kanazawa T., Toda, M., and Tonomura, Y. (1965), *J. Biochem. (Tokyo) 57*, 627.

Imamura, K., Tada, M., and Tonomura, Y. (1966), *J. Biochem. (Tokyo) 59*, 280.

Kanazawa, T., and Tonomura, Y. (1965), *J. Biochem. (Tokyo) 57*, 604.

Kiely, B., and Martonosi, A. (1968), *J. Biol. Chem. 243*, 2273.

Kinoshita, N., Kubo, S., Onishi, H., and Tonomura, Y. (1965), *J. Biochem. (Tokyo) 65*, 285.

Lowey, S., and Luck, S. M. (1969), *Biochemistry 8*, 3195.

Lymn, R. W., and Taylor, E. W. (1970), *Biochemistry 9*, 2975.

Morita, F. (1969), *Biochim. Biophys. Acta 172*, 319.

Nakamura, H., and Tonomura, Y. (1968), *J. Biochem. (Tokyo) 63*, 279.

Rainford, P., Hotta, K., and Morales, M. (1964), *Biochemistry 3*, 1213.

Sartorelli, L., Fromm, H. J., Benson, R. W., and Boyer, P. D. (1966), *Biochemistry 5*, 2877.

Szent-Györgyi, A. G. (1968), *Fed. Proc. 27*, 519.

Tonomura, Y., Nakamura, H., Kinoshita, N., Onishi, H., and Shigekawa, M. (1969), *J. Biochem. (Tokyo) 66*, 599.